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A mammalian protein targeted by G1-arresting rapamycin-receptor complex

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THE structurally related natural products rapamycin and FK506 bind to the same intracellular receptor, FKBP12, yet the resulting

complexes interfere with distinct signalling pathways^{1,2}. FKBP12-rapamycin inhibits progression through the G1 phase of the cell cycle in osteosarcoma³, liver^{4,5} and T cells^{6,7} as well as in yeast⁸, and interferes with mitogenic signalling pathways that are involved in G1 progression^{9,10}, namely with activation of the protein p70^{rac} (refs 5, 11-13) and cyclin-dependent kinases¹⁴⁻¹⁶. Here we isolate a mammalian FKBP-rapamycin-associated protein (FRAP) whose binding to structural variants of rapamycin complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression. Peptide sequences from purified bovine FRAP were used to isolate a human cDNA clone that is highly related to the *DRR1/TOR1* and *DRR2/TOR2* gene products from *Saccharomyces cerevisiae*^{8,17,18}. Although it has not been previously demonstrated that either of the *DRR/TOR* gene products can bind the FKBP-rapamycin complex directly^{17,19}, these yeast genes have been genetically linked to a rapamycin-sensitive pathway and are thought to encode lipid kinases¹⁷⁻²⁰.

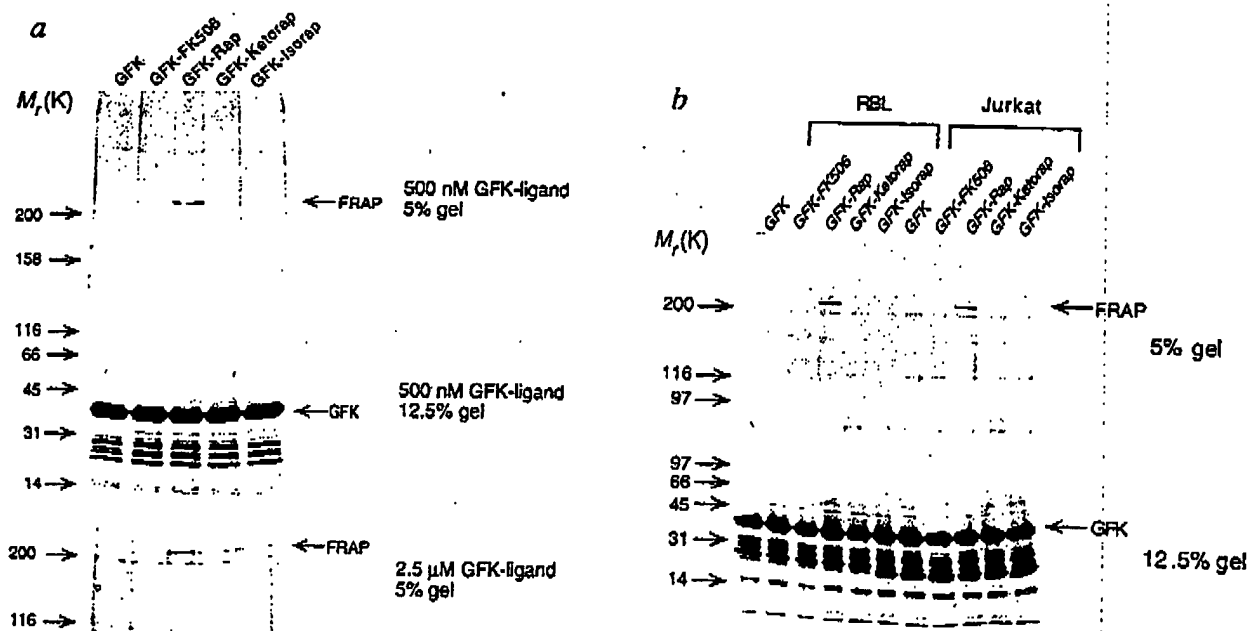


FIG. 1. Identification of FRAP protein in three mammalian cell lines. *a*, GFK alone or individual GFK-ligand complexes were added to MG-63 cell lysates (2×10^7 cells per condition) to a final concentration of either 500 nM or 2.5 μ M and the mixtures incubated for 10 min at 4 °C. Fusion protein complexes were recovered by glutathione-affinity chromatography, and the proteins detected by silver staining after 5% SDS-PAGE. Because of compression, FRAP is not resolved by 12.5% SDS-PAGE, so both 5% and 12.5% gels are shown. The amount of FRAP that was retained by affinity chromatography saturated at concentrations of GFK-Rap greater than 500 nM in these experiments and in others using concentrations of GFK-Rap ranging from 100 nM to 5 μ M (data not shown). *b*, GFK alone or individual GFK-ligand complexes were added to a final concentration of 500 nM to lysates prepared from either 2×10^8 Jurkat T lymphocytes or 10^8 rat basophilic leukaemia (RBL) cells per condition. Lysates were treated as in *a*. FKBP12, but not FKBP13 or FKBP25 (ref. 23) is able to mediate the actions of rapamycin in *S.*

cerevisiae. In addition, we found that YFK188 (ref. 24), an FKBP12 null strain, could be complemented with GFK (P. K. Martin, B. Gladstone, G. Weiss, D. T. Hung, S.L.S., in preparation). Thus the GST appendage of the fusion protein does not preclude binding of the biologically relevant target to the GFK-rapamycin complex in yeast. **METHODS.** MG-63, Jurkat and RBL cells were grown in media containing 10% FBS and lysed at 4 °C in PINT buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μ M Na_2VO_4 , 25 mM 2-glycerophosphate, 0.2 mM PMSF, 1 μ g ml^{-1} leupeptin, 1 μ g ml^{-1} pepstatin A and 2 mM DTT) containing 0.5% Triton X-100. Lysates were clarified by centrifugation at 25,000g, and the Triton X-100 in the supernatant was diluted to 0.33% by adding 0.5 vol PINT buffer. GFK prebound to stoichiometric quantities of FK506, keto-iso- or unmodified rapamycin was added to lysates as described. Each condition was then passed through a 250- μ l glutathione-Sepharose column, which was washed with PINT buffer containing 0.5 M NaCl and 0.3% Triton X-100.

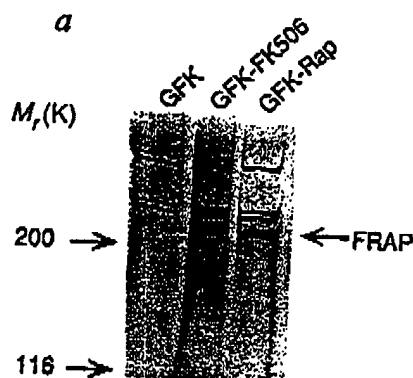


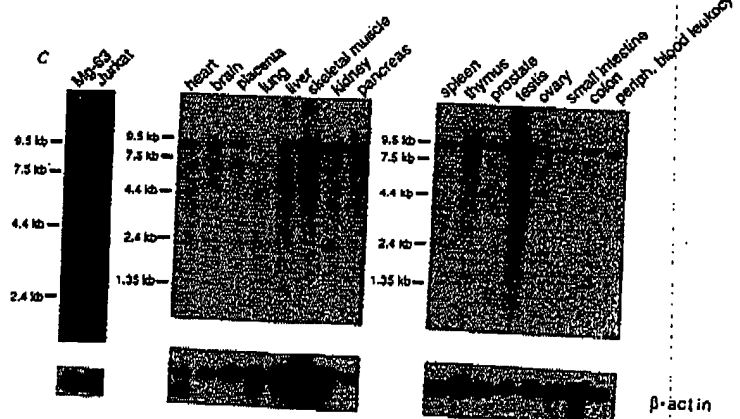
FIG. 2 Purification of FRAP from bovine brain and cDNA cloning of human FRAP. **a**, Fivefold-enriched bovine FRAP (S-column eluate; see below) was conditioned with [100 nM], glutathione-S-transferase-FKBP12 fusion protein (GFK), GFK-FK506 or GFK-Rap. Complexes with fusion proteins were recovered by glutathione-affinity chromatography and detected as described in Fig. 1 legend. We also found FRAP in bovine liver and thymus. **b**, Predicted translational product of the human FRAP cDNA clone. Bovine FRAP peptide sequences aligned to human FRAP are indicated by underlined segments. In the reading frame shown translational stop codons were not encountered upstream of the initiating methionine. **c**, Northern blot analysis of human tissue, Jurkat T cell and MG-63 cell poly (A)⁺ RNA. The Jurkat/MG-63 and multiple tissue Northern blots (Clontech) were hybridized with ³²P-labelled probes derived from the 182 bp PCR fragment and the 5.5 kb clone (text), respectively. Hybridization to human β -actin probe is shown as an internal control for loading.

METHODS. Bovine FRAP was purified by grinding 900 g of bovine brain in blender with 1 litre of PIP (0.3% Triton X-100, 50 mM sodium phosphate, pH 7.2, 2 mM EDTA, 2 mM EGTA, 25 mM NaF, 100 μ M Na₃VO₄, 25 mM 2-glycerophosphate, 1 mM PMSF, 1 μ g ml⁻¹ leupeptin, 1 μ g ml⁻¹ pepstatin A, 1 mM benzamide and 2 mM DTT). The homogenate was centrifuged at 25,000g and the supernatant (20 g total protein) was loaded onto a 1 litre S-Sepharose (Pharmacia) column. The column was then washed with PIP and eluted with PINP (PIP with 1M NaCl). GFK-rapamycin was added to the pooled eluate to a final concentration of 100 nM and recovered by glutathione-affinity chromatography. FRAP was resolved by SDS-PAGE and transferred to PVDF. Following digestion with trypsin or endoproteinase Lys-C (Boehringer Mannheim) bFRAP peptides were microsequenced²³. The Jurkat T cell cDNA library (Stratagene) was constructed through random and oligo dT priming of cytoplasmic oligo dT purified RNA (ref. 25). cDNA screening, Jurkat and MG-63 RNA isolation and northern blotting and were performed by procedures similar to those previously described²³. A 182 bp fragment was amplified from a human brain stem library (Stratagene) and labelled by incorporation of ³²P-dCTP in the course of reamplification by PCR. The sequences were analysed using BLAST (ref. 26) and the University of Wisconsin GCG (ref. 27) software. The human FRAP cDNA sequence has been submitted to Genbank.

We used two structural variants of rapamycin, 16-keto-rapamycin (S. D. Meyer and S.L.S., manuscript in preparation) and 25,26-iso-rapamycin²¹, to identify any biologically relevant targets of the FKBP-rapamycin complex. Both variants bind tightly to human FKBP12, as shown by their ability to inhibit rotamase activity of the recombinant protein (*K_i* values were 0.2 nM for rapamycin⁶, 2 nM for keto-rapamycin, and 0.1 nM for iso-rapamycin). But the variants are about two orders of magnitude less potent than rapamycin in preventing the progression through G1 of MG-63 human osteosarcoma cells. The values of IC₅₀ (half-maximal inhibitory concentration) estimated from dose-response curves are 0.1 nM, 7.5 nM and 50 nM for rapamycin, keto- and iso-rapamycin, respectively. Thus the complexes of iso- and keto-rapamycin with FKBP12 should bind to

b

1	MLGTGPAAT	TAATSSWVS	VLQPFASGLK	SRNEETRAKA	AKELQHYVM	50
51	ELRENSQES	TREYDOLNH	TEFLYSSSDQ	NERKGGILAI	ASLIGVEGN	100
101	ATRICRFANY	LRLNLPNDP	VYVEMASKAI	GRLAMGDTF	TAETVEFEVK	150
151	RALNLGADR	NECARNAVL	VLRELATYVP	TFFPQVQVPP	FDNIFVAVND	200
201	PKQATREGAV	AALRACILIT	TQREPKEHQK	PQWYHTFEE	AEKGFDTLA	250
251	KEKGNRRDR	IRGALLILNE	LVRISSEMEG	RLREEMEEIT	QQQLVHDQYC	300
301	KQLMGCEGTP	RHITPTSEFO	AYQPQQSNAL	VGLLYSSHQ	GLNGFCTSPS	350
351	PAKSTLVESE	CCROLMEEKF	DQVQWVLC	RNSKNSLTQ	TLNLPLRLA	400
401	AFRPSAFTDT	QYLDPTNNHY	LSCYKKEKER	TAAFOALGL	SVAVRSEFVK	450
451	YLPRLVDIIR	AALPKDFAH	KRQKAMQVQA	TVFTCSMLA	RAMPQIQDD	500
501	IKELLEPLHA	VGLSPALTAV	LYDLSRQIQP	LKKQZQDGL	KHLSLVLMHX	550
551	PLRHPGMPKG	LANQLASPL	TTLPASQVQ	SITLALRTLG	SFEPEGHSLT	600
601	QVYKHCADHF	LNSENKEIKM	EAARTCSRLL	TPSHLTSGH	AHYVSTQAVQ	650
651	VYADVLSKLL	VYGITDPOPO	IRYQVLSLSD	ERFDALIAQ	ENLQALFVAL	700
701	NQVFEIREL	AICTVGRLLS	MNPAPVMPFL	RKMLIQILTE	LEHSGTGRIZ	750
751	EQSARMLCHL	VSNAPRLIRP	YMEPTLKALI	LKLIKDPDPD	KPGVINNVLA	800
801	TIGELAQVSG	LEWRKVDDEL	FIINDHLQD	SSLLAKQVVA	LWTLQQLVASI	850
851	TGYVPEYPRK	YPTLLEVLIN	FLKTEQNGY	KREAIRYLGL	LGALDPYKHK	900
901	VHTQIDQSR	DASAVLSSES	KSQSSQSDYS	TSMLVNHGN	LPDEFYPAVI	950
951	SNVLMRIFR	DOSLSWHTM	VQAITTFIFK	SLGLKCVQFL	PQVNPFTLWV	1000
1001	IRVCDGAIRE	FLFQQLMLV	SVFKSHIRPY	MOEIVTLNRE	PWVMTSIOQS	1050
1051	TIILLIEQIV	VALGGEFKLY	LPQITPHMLR	VFMHNSPGR	IVSILKLAZI	1100
1101	QLFGANLDQY	LHLLPRTVY	LFDAPAPLP	SRKALETVD	RLTESLQETD	1150
1151	YASRIINPIY	RILDOSBELR	STAMDTLSL	VFLGKKYQI	FPMVNVKLV	1200
1201	RHRINHOYD	VLICRIVKQY	TLDEFEDD	TYOHRMLRSQ	OGDALASGV	1250
1251	ETCPMKKLHV	STINLOKANG	AABRVSKDDW	LEWLRSLSE	LLKSSSSPSL	1300
1301	RSCRALAQAY	NPMARDLFMA	AFVSCWSELN	EDQOEDTRS	TELALTSQDI	1350
1351	AEVTQLNLN	AEFHEHSDKG	PLPLRDDNGI	VLLGERAAKC	RAYAKALYK	1400
1401	ELEEQKQPTP	AILESISLIN	NKLQOPEAAA	GVLLEYAMKE	GLEIGATNY	1450
1451	EKLHWEEDAL	VAYOKKNOTN	KDDPELMILGR	MKCLLEALGEW	GQLHQQCEK	1500
1501	WTVLNDETQA	KMARMAAAA	WGLGOWDSME	EYTCOMPRDT	HOGAFYRAVL	1550
1551	ALHQDLFSLA	QOCIDKAROL	LDALETAMAG	ESYSRAYCAN	VSCMLSELE	1600
1601	EVIQYKLVEP	RREIRIQIWN	ERLQCCQRIY	EDWOKILMVR	SLVSPHEMD	1650
1651	RDLKLYASLC	KSGRLALAH	KTLVLLQVD	PSRLQHPLE	TVHPQVYAT	1700
1701	MKNWKSARK	ZDAFQMHQF	VQTHQQAQHQ	AIATEDQCHK	QELHKLMAK	1750
1751	FLKLGENQLH	LQGINESTIP	KVLQYSSAAT	EHDRSKYKAN	HARAVNMFEA	1800
1801	VLYYKHQDJA	RDEKKLRLHA	SGANITNATT	ATTAATTAAT	TASTEGSNSE	1850
1851	SEAESTENSP	TPSPLOKKYT	EDLSKTLIMY	TVPAVQGFRR	SISLSRGNHL	1900
1901	QOTLRVLTW	PDYGNHPOVN	EALVEGVKAT	QZOTVNLQVP	QLARIOTPR	1950
1951	PSKRLILHQL	LTDICRYHNPQ	ALTYPLTVAS	KSTTARHVA	ANKILKNMCE	2000
2001	HSKTLVQAM	MYSEELIRVA	ILWHWHMHG	LEASRIYFC	ERVRKMFVE	2050
2051	LEPLHAMMER	GPQTLKETSE	NOAYGDLNE	AQEWCRKYNK	SGMKDLTQA	2100
2101	WOLYHVFR	ISKQLPQLTS	LELQVYSPKL	LMDRLLEAV	PCTYDHPDPT	2150
2151	TRISTIASPI	QVITTSKQRP	KLTIMGSGNH	EFVFLKAGE	DLRQDERVQ	2200
2201	LFCLVNTLLA	NDPTSLKXNL	SIQYAVIPL	STNSGLGW	PHCDTHALI	2250
2251	RDYREKKKIL	LNTEHRLML	MAPOYDHLT	MQVVEVEEA	VNNTAGDRA	2300
2301	KLLRLKSPSS	EVNFORTNY	TRSLAVNSHV	GYILGLGRH	PSNMLDRIS	2350
2351	GKILHIDFGD	CFEYANTRK	FPEKIPFRLT	RMLTNAMET	GLDGNVRET	2400
2401	HTVMEVLRN	KDSYMAVLEA	FYDPLNMR	LMQNTKGNK	BSRTDSDYS	2450
2451	AGOSVETLDS	VELGEPAHKK	IGTTVPESIH	SEIGDGLVYK	FALNKATQI	2500
2501	INRVKRLTG	RDFSDDTLD	VPTQVELLIK	QATSHENLCQ	CTYGCWPF	2549



the FKBP12-rapamycin target less effectively than FKBP12-rapamycin itself.

A fusion protein of glutathione-S-transferase with FKBP12 (GFK) was used to identify candidates for the biologically relevant targets of FKBP12-rapamycin. MG-63 cells were lysed by detergent and complexes of GFK-rapamycin, GFK-FK506 or GFK alone were added individually to clarified lysate at a final concentration of 500 nM or 2.5 μ M (Fig. 1a). A protein of approximate relative molecular mass 220,000 (*M_r* ~ 220K) was detected in the GFK-rapamycin sample by SDS-PAGE and silver staining (Fig. 1a, lane 3). This FKBP-rapamycin-associated protein (FRAP) was not retained with GFK-FK506 or GFK alone (Fig. 1a, lanes 1 and 2). No other rapamycin-specific proteins were detected by silver staining (Fig. 1a) or by a similar